Red shift of the purple membrane absorption band and the deprotonation of tyrosine residues at high pH

Origin of the parallel photocycles of trans-bacteriorhodopsin

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ABSTRACT At high pH (> 8) the 570 nm absorption band of all-*trans* bacteriorhodopsin (bR) in purple membrane undergoes a small (1.5 nm) shift to longer wavelengths, which causes a maximal increase in absorption at 615 nm. The pK of the shift is 9.0 in the presence of 167 mM KCl, and its intrinsic pK is ~8.3. The red shift of the *trans*-bR absorption spectrum correlates with the appearance of the fast component in the light-induced L to M transition, and absorption increases at 238 and 297 nm which are apparently caused by the deprotonation of a tyrosine residue and red shift of the absorption of tryptophan residues. This suggests that the deprotonation of a tyrosine residue with an exceptionally low pK ($pK_a \approx 8.3$) is responsible for the absorption shift of the chromophore band and fast M formation. The pH and salt dependent equilibrium between the two forms of bR, "neutral" and "alkaline," bR ↔ bR_a, results in two parallel photocycles of *trans*-bR at high pH, differing in the rate of the L to M transition. In the pH range 10–11.8 deprotonation of two more tyrosine residues is observed with pK's ~10.3 and 11.3 (in 167 mM KCL). Two simple models discussing the role of the pH induced tyrosine deprotonation in the photocycle and proton pumping are presented.

It is suggested that the shifts of the absorption bands at high pH are due to the appearance of a negatively charged group inside the protein (tyrosinate) which causes electrochromic shifts of the chromophore and protein absorption bands due to the interaction with the dipole moments in the ground and excited states of bR (Stark effect). This effect gives evidence for a significant change in the dipole moment of the chromophore of bR upon excitation.

Under illumination alkaline bR forms, besides the usual photocycle intermediates, a long-lived species with absorption maximum at 500 nm (P500). P500 slowly converts into bR_a in the dark. Upon illumination P500 is transformed into an intermediate having an absorption maximum at 380 nm (P380). P380 can be reconverted to P500 by blue light illumination or by incubation in the dark.

INTRODUCTION

In this paper we describe the pH and salt dependence of some spectral and photochemical properties of *trans*-bacteriorhodopsin in suspensions of purple membrane at pH 8–11. In earlier studies (Shkrob and Rodionov, 1978; Ovchinnikov et al., 1980; Druckmann et al., 1982; Muccio and Cassim, 1979; Aldeshev and Efremov, 1983; Scherrer and Stoeckenius, 1984) no significant changes in the color of the pigment were found in this pH range. However, Maeda et al. (1986, 1988) noted a small red shift of the absorption spectrum upon raising the pH above eight; this change will be studied in detail below. At pH > 11.3–11.8 major changes occur, the reversible deprotonation of the chromophore and subsequent alkaline denaturation, and bR turns into a state with absorption maximum at ~480 nm (pK ≈ 12). According to resonance Raman data (Druckmann et al., 1982) this state has an unprotonated Schiff base chromophore. At pH > 11.8, the absorption maximum shifts to ~370 nm and purple membrane structure undergoes irreversible changes (Shkrob and Rodinov, 1978; Muccio and Cassim, 1979; Druckmann et al., 1982).

Upon increasing the pH, the reactions of the photocycle undergo prominent changes. At pH > 8 or 9 (depending on the salt concentration) the kinetics of M formation becomes faster (e.g., Ort and Parson, 1978; Rosenbach et al., 1982; Hanamoto et al., 1984; Liu, 1990) due to the appearance and progressive increase in the amplitude of two fast components with the rise time 6 and 0.4 μs (Liu, 1990), which replace a slower 85 μs component which predominates at neutral pH. The pK of this transition is 10.3 in water (Rosenbach et al., 1982) and 9.6 in the presence of 0.1 M K^+ (KH_2PO_4) (Hanamoto et al., 1984). It was proposed that the changes in the kinetics of M formation are caused by deprotonation of an amino acid residue (maybe a tyrosine) in the vicinity of the chromophore (Rosenbach et al., 1982; Hanamoto et al., 1984). The state of other amino acid residues also affect the kinetics of bR photoconversion. For example, in the bR mutant in which Asp85 was replaced by glutamic acid, fast components of M formation appeared even at neutral pH (Butt et al., 1989). Substitution of Asp96, which appears to function as an internal donor of protons to the Schiff base, causes a

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drastic increase in the lifetime of the M and N intermediates at high pH (Butt et al., 1989, Holz et al., 1989). Thus, studies of the pH dependence of the reactions of the photocycle provide important information on the interaction of the chromophore and the Schiff base with the apoprotein.

There is one more aspect of this interaction which may throw some additional light upon the nature of the early photochemical events taking place upon excitation of the chromophore. Data obtained by fluorescence (Sineshschekov et al., 1981; Balashov et al., 1988) and subpicosecond spectroscopy (Mathies et al., 1988) indicate that isomerization of the chromophore takes place already in the excited state of the pigment. It has been suggested that during excitation of the chromophore redistribution of electron density occurs which results in a decrease of the order of the double bonds and an increase in that of single bonds, thus, permitting isomerization around double bonds in the excited state (Honig and Ebrey, 1974).

Mathies and Stryer (1976) determined the change in electric dipole moment ($\Delta\mu$) of free all-*trans* retinal upon excitation to be 12 Debye. Recently Birge and Zhang (1990) provided evidence that the electric dipole moment of bR is ~13.5 Debye. One possible consequence of this large dipole moment change would be the observation of small changes in the position of the purple membrane absorption band produced by a strong electric field. Due to the existence of ionizable groups on the surface of the purple membrane and inside the protein (Ovchinnikov et al., 1979, 1980; Henderson et al., 1990) one may expect that changes in the surface charge could change the electrostatic field inside the protein and due to the Stark effect (called electrochromism in the case of polyatomic molecules) result in a small shift (1–5 nm, Balashov et al., 1987) of the absorption band. The magnitude and the direction of the shift directly depends on the magnitude and mutual orientation of the vectors of the electric field, **F**, and the change of the dipole moment $\Delta\boldsymbol{\mu}$ (Bücher and Kuhn, 1970).

In the present study we measured the absorption spectrum of light-adapted purple membrane at different pH's at room and low temperature. A small (1.5 nm) shift of the absorption spectrum to longer wavelengths was observed at high pH. This is ascribed to the formation of a new form of bR, alkaline bR, bR_a. The formation of bR_a correlates with (*a*) an absorbance increase at 238 nm apparently caused by deprotonation of a tyrosine residue and (*b*) an increase in the fraction of the pigment yielding the M intermediate with a fast formation time. These observations support the idea of two parallel photocycles of bR at high pH.

The nature of the red shift is discussed in terms of an electrostatic interaction of a negatively charged tyrosinate residue with the chromophore and nearby tryptophan residues. In the framework of this model the shift of the absorption bands provides evidence for a significant shift of electron density in the chromophore of bacteriorhodopsin during the transition into its excited state.

METHODS

Halobacterium halobium, strain S9, was grown and purple membrane isolated as described previously (Becher and Cassim, 1975). Absorption spectra were measured on a Cary-Aviv 14 spectrophotometer (Aviv Associates, Lakewood, NJ) by digital recording with 1 nm steps and a spectral bandwidth of 1 nm in visible range and with a 0.5 nm step in ultraviolet (UV). The difference and derivative spectra were obtained using the utilities of the spectrophotometer.

Because cooling of the water-glycerol (1:2) suspensions of purple membrane results in sharpening of the absorption band, absorption spectra were measured at −165°C in a homemade cryostat similar to that described previously (Litvin et al., 1975). It consists of a brass cuvette (pathlength 2 mm) with windows made of acrylic plastic attached to the cold finger cooled with liquid nitrogen. The cuvette and the finger with liquid nitrogen were placed inside the quartz Dewar which was fixed in a holder in Cary-Aviv spectrophotometer. The system was cooled by addition of liquid nitrogen inside the cold finger. The actual temperature in the cuvette was monitored by a copper-constantan thermocouple immersed into the sample and attached to a Cole-Parmer Digi-Sense thermometer.

Light adaptation of purple membrane was made by the light from a slide projector passed through a light guide and cut-off filters. In the case of water suspensions of purple membrane and room temperature measurements, light-adaptation was produced by illumination at wavelengths > 500 nm. For water-glycerol suspensions and low temperature measurements illumination at 490–540 nm was used (~10 W/m^2) and the sample was light adapted twice, 20 min at room temperature and then 15 min at 5°C. At 5°C the spontaneous transformation of *trans*-bR into 13-*cis*-bR becomes very slow which permits the maximum amount of bR to be converted into the trans-form. After a 5 min incubation in the dark at 5°C (to allow all the photocycle intermediates to relax back to bR) the sample was slowly cooled to −160°C. Usually the sample froze with no, or 1–2, cracks which slightly increased the light scattering particularly at wavelengths shorter than 500 nm.

The "Good" buffers (MES, Hepes; Good et al., 1966), tris and bicarbonate were used to maintain the pH. The pH of the sample was measured at 20°C with a glass electrode and pH 62 potentiometer (Radiometer America, Inc., West Lake, OH). The pH of the suspensions is temperature dependent. According to Maurel et al. (1975) the pH of tris buffer in water-glycerol (50:50 vol/vol) shifts from 8 to ~10 upon lowering the temperature from 20 to −40°C. This effect was important in our measurements because it allowed us to light adapt at moderate pH (8.0–8.5) and by subsequent cooling increase the pH of the suspension to 10. This prevented formation of the photoproducts peculiar to purple membrane at high pH during light adaptation. The temperature coefficients of Hepes and MES buffers are two to three times smaller than that of tris, and thus, the change in pH of the samples in these buffers was not that large.

RESULTS

pH dependence of the absorption spectrum at low temperature

Fig. 1 *A* shows the low temperature (−160°C) absorption spectra of water-glycerol suspensions of purple membrane in the presence of 16 mM MES (pH 5.5 at 20°C) and in the presence of tris (pH 8.0 at 20°C). One can see that the spectrum at alkaline pH is slightly shifted (≈ 1.5 nm) to longer wavelengths. At −160°C the maximum in the absorption spectrum of a suspension of light-adapted purple membrane in MES is at 576 nm, whereas the maximum in tris is at 577.5 nm. At −160°C the difference absorption spectrum of light-adapted purple membrane in the presence of tris minus light-adapted purple membrane in the presence of MES has a maximum at 615 nm and a minimum at 540 nm (Fig. 1 *B*). The difference maximum is close to the maximum of the first derivative of the absorption spectrum (taken with a minus; curve not shown), which is exactly what is expected if only a shift of the absorption band occurs (Donovan, 1969a).

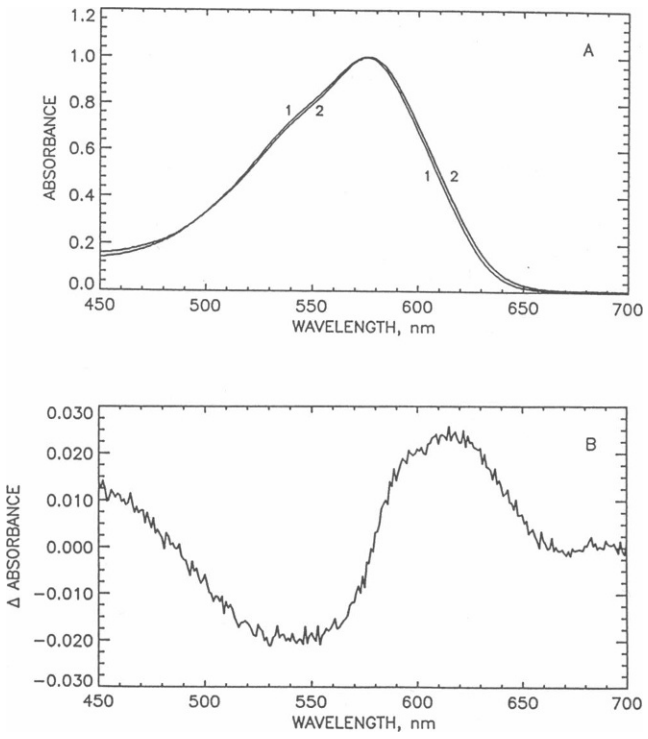

FIGURE 1 (*A*) Absorption spectra of water-glycerol (1:2) purple membrane suspensions that were light-adapted at 20 and 5°C and then frozen to −160°C in the dark: 1, in the presence of 16 mM MES + 1 mM NaOH, pH 5.5 at 20°C; 2, in the presence of 20 mM tris, pH 8 at 20°C. The spectra were normalized at their long wavelength maxima. (*B*) Difference absorption spectrum "2–1".

Illumination of purple membrane at 500 nm produces the bathoproduct K. The spectral changes accompanying K formation were basically the same for frozen alkaline and acid suspensions. However, minor spectral differences were present. The isosbestic point between bR and K in the alkaline suspension was at 594 nm, whereas in the acid suspension it was at 592 nm. The concentration of 13-*cis* bR was less than five percent in both acid and alkaline suspensions (which was estimated from the amount of the bathoproduct of 13-*cis* bR; Tokunaga et al., 1976; Balashov et al., 1987, 1988) and could not cause the shift of the spectrum. The shift of the isosbestic point is caused by the shift of the absorption spectrum of initial bR.

pH induced absorption changes in the chromophore absorption band of purple membrane at room temperature

To find out whether the red shift of the bR absorption band at high pH occurs at room temperature we studied pH induced absorbance changes of light-adapted purple membrane at 20°C. The membranes in 167 mM KCl were light-adapted at pH 7–7.4, 15–20°C, with yellow light (500–700 nm; model CS 3-70; Corning Glass Inc., Corning, NY), and then the pH was adjusted in the dark by the addition of 10 μl aliquots of 0.01–1 M NaOH. The alkalinization of the sample was done in the dark to avoid the light-induced formation of the photoproducts P500 and P380 peculiar for purple membrane photoconversion at high pH (see below).

The absorption spectra of suspensions of light-adapted purple membrane in 167 mM KCl were measured at 20°C at pH 7.4, 8.2, 8.6, 9.2, 9.9, 10.7, 11.4, and 11.8 (Fig. 2 *A*). The spectra were measured within 3 min after adjusting the pH. During this time dark-adaptation was insignificant. Alkalinization of the suspension caused a decrease in absorption at 570 nm and an increase at 450 nm. The difference spectra (pH_i–pH7.4) have a cross-over point ~502 nm in the pH range 8.2–9.9. At higher pH (11.4 and 11.8) the cross-over point shifts to 515 nm. The decrease in the absorption in the main band is 8% at pH 10.7, and it reaches 23% at pH 11.8. These spectral changes are completely reversible up to pH 11.

The difference spectra (Fig. 2, *B* and *C*) between the high pH spectra and the spectrum at pH 7.4 have a negative band at 568 nm and two positive bands, at 630 and 455–460 nm. The shape of the difference spectra changes with pH, which indicates that more than one species is formed at high pH. The absolute amplitude of the 630 nm band increases with increasing pH up to pH 9.9 and then decreases. The amplitude of the increase at 630 nm relative to the decrease at 568 nm [$\Delta A(630)$/

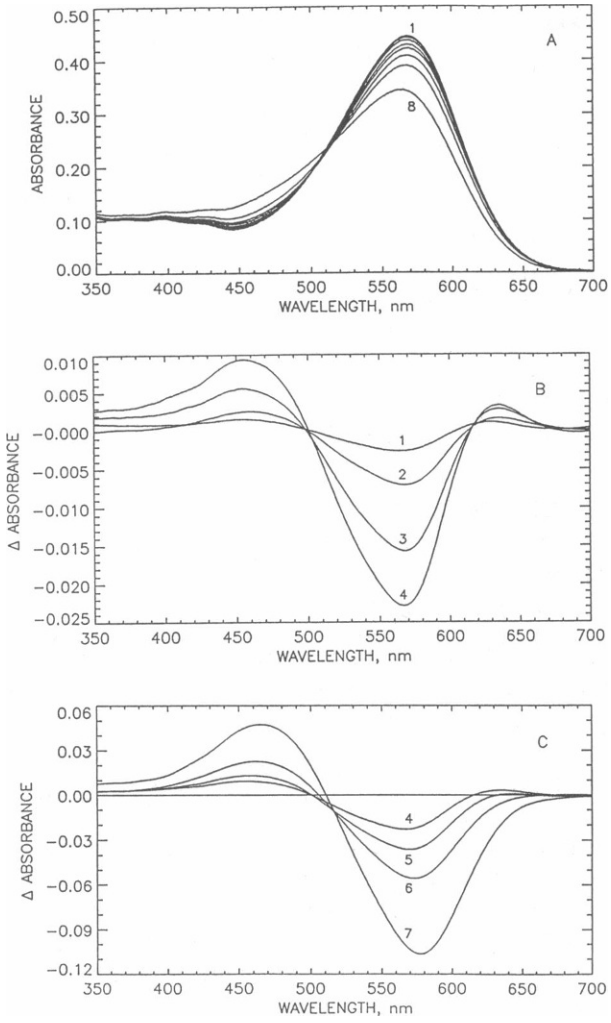

FIGURE 2 (*A*) Absorption spectra of a water suspension of purple membrane in 167 mM KCl as a function of pH at room temperature. The purple membrane suspension was light-adapted at pH 7.4 and then adjusted to the certain pH in the dark by the addition of NaOH. 1–7 (in the order of decrease of absorbance at the maximum): 1, pH 7.4; 2, pH 8.2; 3, pH 8.6; 4, pH 9.2; 5, pH 9.9; 6, pH 10.7; 7, pH 11.3; 8, pH 11.7. (*B* and *C*) Difference absorption spectra of light-adapted purple membrane in 167 mM KCl at pH_i minus that at pH 7.4. 1, pH_i 8.2; 2, pH_i 8.6; 3, pH_i 9.2; 4, pH_i 9.9; 5, pH_i 10.7; 6, pH_i 11.3; 7, pH_i 11.7. Optical density of the initial sample at 568 nm was 0.45 (at pH 7.4), pathlength of the cuvette 5 mm.

$\Delta A(568)$] is highest at pH 8.2 (0.58) and drops at higher pH (e.g., 0.05 at 10.7). The 630 nm band is not observed in the difference spectra at pH 11.4 and 11.8 (Fig. 2 *C*). In contrast to this pH behavior, the amplitude of the absorption changes at 450 nm, which are caused mainly by the transformation of bR into a species absorbing at shorter wavelengths (P480, see below), increases monotonically with increasing pH. The different pH dependences of the amplitudes of the two bands clearly indicate that the shift of the spectrum to longer wavelengths, which is the cause of the 630 nm band, and transformation into P480 are two different processes. We propose that at first the transformation of bR into the alkaline purple form bR_a occurs, $bR \rightarrow bR_a$. Then bR_a comes into a pH dependent equilibrium with a species absorbing at shorter wavelengths (P480). At pH > 11.0, the minimum in the difference spectra shifts slightly to longer wavelengths.

The pH dependence of the shift of the 568 nm absorption band to the red can be estimated by normalizing the spectra at their 568 nm maxima (in order to compensate for the partial transformation of bR into P480) and measuring the amplitude of the difference spectra. Fig. 3 shows the difference spectra thus obtained. From the pH dependence of the amplitude of the absorbance changes at 615 nm, the pK of the red shift was estimated to be 9.0 in 167 mM KCl. At pH 9.9 the shift is almost complete (it reaches 90% of saturation).

Decomposition of the pH induced absorbance changes into two components, corresponding to the formation of bR_a and P480

The different pH dependences of the red shift and transformation of bR into P480, enable us to decompose the pH induced absorption changes into two spectral components related to each process. Because the concentration of the red-shifted species (bR_a) was the same at pH 9.9 and pH 10.7 (compare the corresponding curves in Fig. 3), the absorbance changes between pH 9.9 and 10.7 were caused only by the transformation of bR into P480, and thus, the difference spectrum "pH 10.7 minus

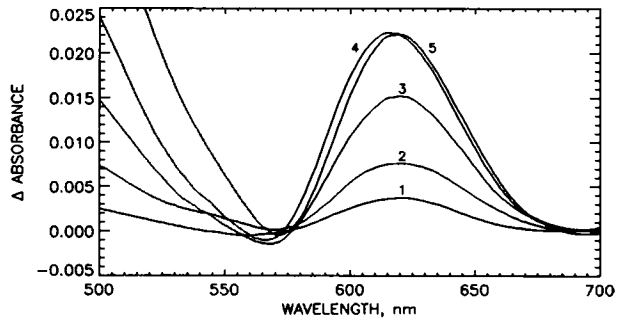

FIGURE 3 Difference spectra of pH_i minus pH 7.4. Spectra at pH_i were first normalized at the maximum (568 nm): 1, pH_i 8.2; 2, pH_i 8.63; 3, pH_i 9.2; 4, pH_i 9.9; 5, pH_i 10.7.

pH 9.9" reflects only this transformation. By adding the spectrum of bR with an appropriate scaling factor to the difference spectrum of pH 10.7 − 9.9, the spectrum of P480 (Fig. 4, *curve 1*) was obtained. It has a maximum at 478 nm and a broad shoulder at 550 nm which may belong to P480 or to some other species. The maximum extinction of P480 is ~0.55 that of bR. The pK of the transformation of bR_a into P480 is estimated to be 12.1 in 167 mM KCl which is close to the previous estimate of 12 in 20 mM NaCl of Scherrer and Stoeckenius (1984) measured in dark-adapted membranes.

The difference spectrum corresponding to the red shift was obtained by subtracting the difference spectrum between P480 and bR (pH 10.7 − 9.9 multiplied by a scaling factor, Fig. 4 *B*) from the spectrum pH 9.9 − 7.4. The resulting difference spectrum (Fig. 5, *curve 1*) has a positive band at 610 nm, a cross-over at 570 nm, and a negative band at 537 nm. Its shape is close to the analytical difference spectrum obtained by subtracting the spectrum of initial bR from the spectrum shifted by 40 cm^{-1} (1.4 nm at 570 nm) to the red (Fig. 5, *curve 2*).

The spectrum of bR_a can be obtained by adding of "bR_a − bR" difference spectrum (Fig. 5, *curve 1*) to the spectrum of bR. This procedure assumes that all the pigment is capable of transformation into bR_a. This suggestion is supported by the correlation of the amplitude of the shift and the fraction of bR undergoing fast M formation (next section). The calculated spectrum of the alkaline form of bR, bR_a, has an absorption band similar to bR but is shifted 1.4 nm to the red (Fig. 4, *curve 2*). The maximal extinction of bR_a is apparently the same as that of bR.

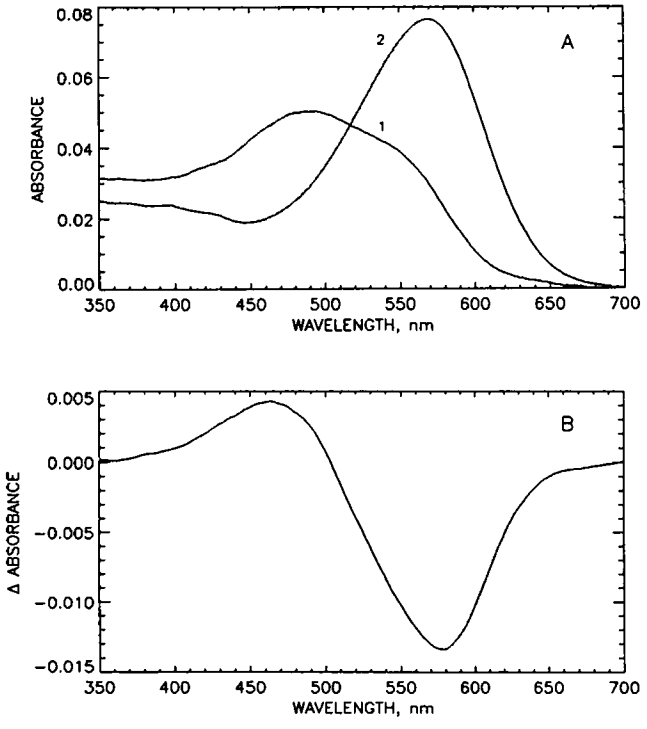

FIGURE 4 (*A*) Absorption spectra of bR_a and P480 calculated as described in the text. (*B*) Difference spectrum corresponding to the transition of bR_a to P480, obtained as a difference between spectra of light-adapted purple membrane measured at pH 10.7 and 9.9.

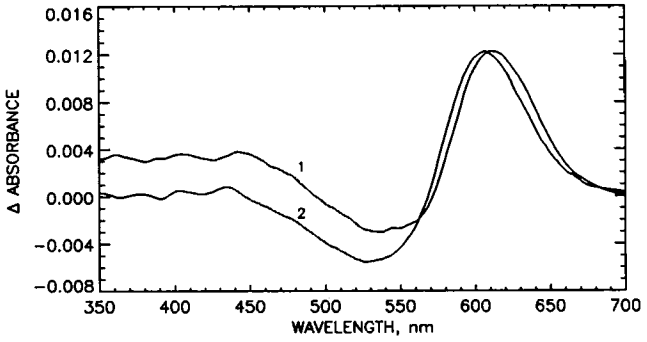

FIGURE 5 The difference spectrum corresponding to the transformation of bR into bR_a (1), and an analytical spectrum corresponding to a shift of the bR absorption band by 1.4 nm to the longer wavelengths (2). Spectrum 1 was obtained by subtracting absorption changes associated with the conversion of bR_a into P480 with appropriate scaling factor from the absorption changes "pH 9.9 minus pH 7.4." Spectrum 2 is proportional to $dA/d\nu = -(dA/d\lambda)\,\lambda^2$.

From the basis spectra of bR_a and P480, we calculated their fractions of the total pigment along with that of neutral bR at different pH. The fraction of P480, f P480, was calculated from the decrease of absorption at 568 nm: $f\text{P480} = 1.3\ \Delta A568/A_o$, where 1.3 was determined from Fig. 4 as the ratio of absorbance of bR (bR_a) at 568 nm and the difference in absorbance between bR and P480 at 568 nm, $\Delta A568$; A_o is the absorbance of light-adapted purple membrane at 568 nm at pH 7.4. The fraction of bR + bR_a was equal to 1 − fP480. The fraction of bR_a was obtained from the pK plot of the amplitudes of the difference spectra shown in Fig. 3. The results are summarized in Table 1. One can see that at pH 9.9 − 10.7 the concentration of bR_a reaches a maximum (≈90%) and then decreases due to the transformation of bR_a into P480.

A comparison of the difference spectra taken at the same pH in light-adapted and dark-adapted membranes shows that the amplitude of the 630 nm band in the dark-adapted membrane is only 0.5–0.4 of that in light-adapted membrane, which correlates with the fraction of *trans*-bR in the dark-adapted samples. This result indicates that the 630 nm absorption band is due mainly to the shift of the absorption spectrum of *trans*-bR.

TABLE 1 **Fractions of the "neutral" form of bacteriorhodopsin, bR, the alkaline form, bR_a, the very alkaline form, P480, and the number of tyrosinate residues per molecule of bR as a function of pH in light-adapted purple membrane in 167 mM KCl at 20°C**

pH	bR	bR_a	P480	δTyr⁻/mol bR
7.4	0.99	0.01	—	—
8.2	0.84	0.15	0.01	0.14
8.6	0.68	0.30	0.02	0.32
9.2	0.36	0.60	0.04	0.61
9.9	0.05	0.89	0.06	1.10
10.7	0.01	0.89	0.10	1.94
11.3	0.00	0.84	0.17	2.55
11.7	0.00	0.68	0.33	3.07

Salt dependence of the red shift of the chromophore absorption band

The titration curves of the red shift (measured as the amplitude of absorbance changes at 615 nm in the absorption spectra at different pH normalized at 568 nm) are shown in Fig. 6. At low salt concentration (0.5 mM K^+) the pK is ~10.3. At 0.02, 0.2, and 1.5 M KCl it shifts to 9.6, 9.0, and 8.7, respectively. The decrease in the pK at high salt concentrations is apparently caused by the reduction of the negative surface charge of the membrane by cations (reviewed in Jonas et al., 1990). The pK at the highest salt concentration is close to the "intrinsic pK" of the group responsible for the shift. Using the Gouy-Chapman and Boltzmann equations as described in Koutalos et al. (1990) the intrinsic pK of this group was calculated to be ≈8.3.

Correlation of the red shift with changes in the kinetics of the L–M transition

At high pH the rate of M formation becomes faster (Ort and Parson, 1978; Rosenbach et al., 1982). With an increase in pH the amplitude of the slow rising (85 μs) component decreases, whereas the amplitudes of the fast (6 μs) and very fast (0.3 μs) components increase (Hanamoto et al., 1984; Liu, 1990); see Fig. 7 *A*.

The question arises, is the red shift of the absorption spectrum and the changes in the photocycle kinetics caused by the same pH dependent event, the deprotonation of some group in the protein? If so, one should expect that the pK of the red shift and of the transition from slow to a fast (and very fast) M formation time should coincide. Fig. 7 *B* shows the pH titration curve for the sum of the amplitudes of the fast and very fast M forms in the presence of 150 mM KCl. The pK (9.2) thus obtained is in good agreement with the pK of the shift (9.0 for 190 mM KCl). Both titration curves are well fitted by an $n = 1$ curve, suggesting the deprotonation of a single group is involved. These data indicate that both the red shift of the spectrum and changes in the photocycle kinetics are caused by the deprotonation of

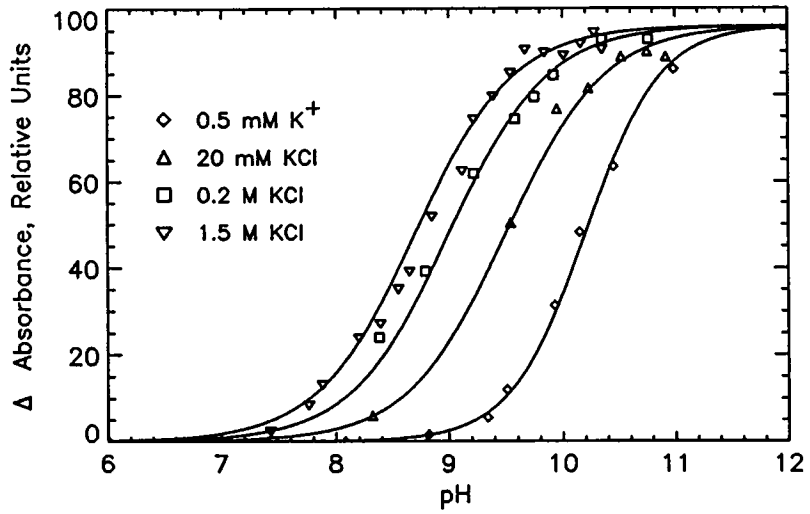

FIGURE 6 Titration curves of the pH-induced red shift (measured by the increase in absorption at 615 nm) at different salt concentrations. The titration curves were obtained from purple membrane samples which were light adapted at pH 7 and also at high pH. This caused the transformation of a part of the pigment to P380 and P500 (see Fig. 10), which slightly affected the pK, shifting it ≈0.2 U to higher values as compared to those obtained when light-adapted purple membrane was not illuminated at high pH. Lines are best fit theoretical curves for an $n = 1$ transition.

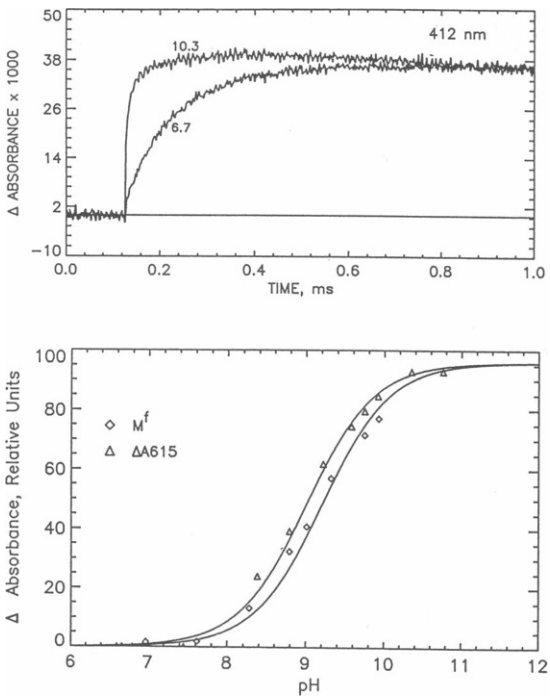

FIGURE 7 (*A*) Kinetics of M formation at pH 6.7 and 10.3 (*top*). (*B*) Titration curve for the amplitude of the fast M formation in 150 mM KCl and the pH induced absorption shift in 190 mM KCl (*bottom*) measured by the increase in absorption at 615 nm. The smooth lines are fit to an $n = 1$ titration with pK of 9.2 and 9.0, respectively.

the same single amino acid residue. As shown by Corcoran et al. (1986), the pK of the transition from slow to fast M formation is also salt dependent. It changes from 10 at low salt (0.2 M NaCl) to 8.6 at high salt (4.5 M NaCl) which is in good agreement with our data on the salt dependence of bR → bR_a transition. The correlation between the fraction of bR in the alkaline form, bR_a, and the fraction of pigment capable of fast M formation suggests that bR_a is the pigment undergoing fast L to M transition. This can be described by the scheme shown in Fig. 8. Intermediates originating from the alkaline form of bR are designated by subscript a.

Correlation of the pH induced absorption changes in the chromophore and protein absorption band

The finding of the alkaline form of bR, bR_a, raises the question whether changes in the protein part of the pigment (e.g., tyrosine deprotonation or a shift of the absorption band of tryptophan residues) can be correlated with the formation of this pigment.

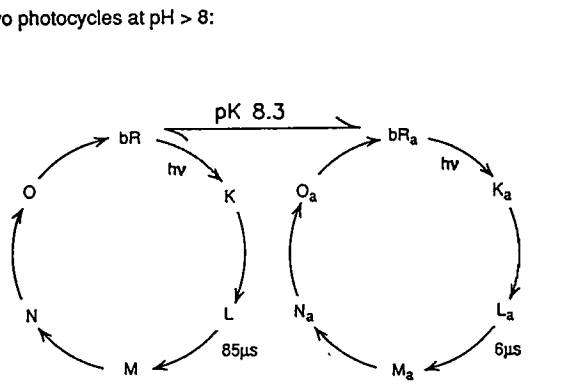

FIGURE 8 Scheme of cyclic reactions of bR at high pH. bR_a, alkaline form of bR (its absorption spectrum is shifted 1.5 nm to the red). The intrinsic pK of the transition from bR to bR_a is ~8.3; it will be higher as the salt concentration decreases from ~4 M NaCl.

In several previous studies (Bogomolni et al., 1978; Efremov et al., 1978; Muccio and Cassim, 1979; Aldashev and Efremov, 1983; Scherrer and Stoeckenius, 1984; Maeda et al., 1986, 1988) the pH induced absorbance changes in the UV range were described. Bogomolni et al. (1978) titrated purple membrane in the dark and found deprotonation of about one tyrosine at pH 10.5 in 2.5 mM NaCl with characteristic bands at 240 and 300 nm. Muccio and Cassim (1979) also observed an increase in absorption at 240 and 297 nm in a water suspension of dark-adapted purple membrane upon increasing the pH. The 297 nm band was attributed to deprotonation of tyrosine residues having a pK of 10.5 in water. The titration curve of the absorbance changes at 240 nm had several inflections, which were interpreted as due to alterations of the environment of several aromatic amino acids. Efremov et al. (1978) and Aldashev and Efremov (1983) ascribed the pH induced absorption changes at 240 and 297 nm to the ionization of a tyrosine residue with a pK of 10.6 ± 0.3 in 10 mM salt. They concluded that in the pH range between 9 and 11.5 only one tyrosine residue deprotonates in the purple membrane. The maximum at 288 nm, seen in the difference spectra, was explained as being due to the hypsochromic shift of the absorption band of tryptophan residues caused by the transition from a hydrophobic into a more polar environment. A similar interpretation of the absorbance changes in the UV was given by Bogomolni et al. (1978) and R. Renthal (personal communication), who proceeding from the amplitude of the absorption changes at 238 and 300 nm estimated that 0.8–1.0 tyrosine residue per bR molecule deprotonates in the purple membrane upon increasing the pH from 7.1 to 10.7 in the presence of 2.5 mM NaCl. Scherrer and

Stoeckenius (1984) and Maeda et al. (1986) titrated purple membrane and noticed that double peaks at 288 and 297 nm resemble the absorption changes produced by perturbation of the tryptophan absorption spectrum. These authors suggested that these peaks are caused by the shift of the spectra of tryptophan produced by the appearance of a negative charge due to the deprotonation of some amino acid residue (tyrosine, Scherrer and Stoeckenius, 1984; a very high pK carboxyl or a lysine, Maeda et al., 1986, 1988).

To investigate the possible correlation of changes in the protein absorption band with the formation of bR_a and P480, we studied the pH induced absorbance changes of light-adapted purple membrane at room temperature simultaneously in the visible and near ultraviolet and compared the amount of bR_a and P480 at different pH with the fraction of tyrosinate residues formed at high pH, which was estimated from the size of the increase of the 240 nm absorption band.

The pH induced absorption changes in the UV (230–320 nm) are shown in Fig. 9 *A*. They are characterized by an intense band appearing near 240 nm and a second band appearing at 297 nm but having structure. The shape of the difference spectra, "pH_i − 7.4" changes with pH. As the pH increases, three different difference spectra arise (Fig. 9 *B*). One of them "pH 10.7 − 9.9" (Fig. 9 *B, curve 2*) can be easily identified as the difference spectrum for tyrosine deprotonation because it practically coincides with the difference spectrum pH 7 − 11 for free tyrosine in solution (Fig. 10 *A*, *curve 2*). The two others have a more complex structure, discussed below.

(*a*) In the range from pH 8.2 to 9.9 the difference spectrum is characterized by a band at 238 nm, two peaks at 287.5 and 296.5 nm, a minimum between them at 290.5 nm and minor shoulders at 277 and 282 nm, (Fig. 9 *B, curve 1*). Within the experimental error the shape of the spectrum does not change between pH 8.2 and 9.9 in 167 mM KCl. The amplitude of the absorption increase at 238 nm (pK = 9.0) correlates very well with the amplitude of the absorbance increase at 615 nm in the normalized difference spectra (pK = 9.2; Fig. 3), caused by the formation of bR_a. The increase of absorption near 240 nm is peculiar to deprotonation of the phenolic OH-group of tyrosine (Donovan, 1969*a*). Using the molar difference extinction coefficient for the tyrosine-tyrosinate absorption changes at 238 nm (11,000 l/mol cm, Donovan, 1969*a*), one can estimate the number of tyrosinates per bR molecule at different pH. We found that the amount of deprotonated tyrosine residues coincide within the experimental error (±5%) with the fraction of bR in the alkaline form, bR_a (see Table 1). This result strongly suggests that the formation of

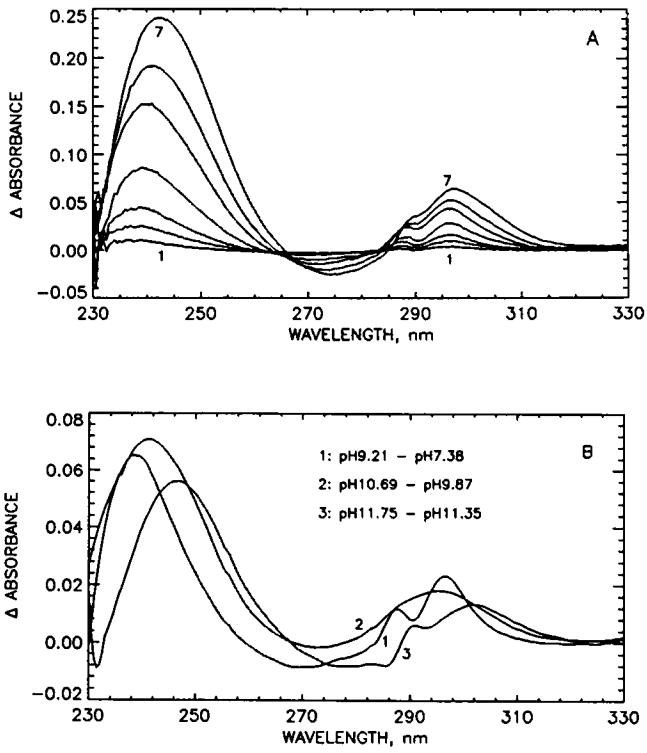

FIGURE 9 (*A*) Difference absorption spectra of light-adapted purple membrane in 167 mM KCl at pH_i minus that at pH 7.4. The membranes were light-adapted at pH 7.4 and then adjusted to pH: 1, pH 8.2; 2, pH 8.6; 3, pH 9.2; 4, pH 9.9; 5, pH 10.7; 6, pH 11.3; 7, pH 11.7. Optical density of the sample at 568 nm was 0.45. (*B*) Difference spectra corresponding to three different pH-induced processes in purple membrane, obtained by subtracting absorption spectra of light-adapted PM measured at two different pH: 1, pH 9.2 minus pH 7.4 (for convenience of comparison this spectrum was multiplied by 1.5); 2, pH 10.7 minus pH 9.9; 3, pH 11.7 minus pH 11.3.

bR_a is caused by the deprotonation of one tyrosine residue.

The ionization of tyrosine is also accompanied by the shift of its 280 nm band to longer wavelengths, which results in the appearance of a broad band near 295 nm in the difference spectrum and a molar extinction coefficient equal to 2,350 l/mol cm (Donovan, 1969*a*), 4.8 times smaller than the 238 nm peak. In the purple membrane the two peaks at 287 and 297 nm can be interpreted as a superposition of the tyrosinate difference absorption spectrum and changes due to the shift of the absorption band of tryptophan residues (Bogomolni et al., 1978; Efremov et al., 1978; Aldashev and Efremov, 1983; Scherrer and Stoeckenius, 1984; Maeda et al., 1986), see Fig. 10. A similar proposal has been made for the somewhat different light-induced changes for bR → M in the UV (see Bogomolni, 1980; Czégé et

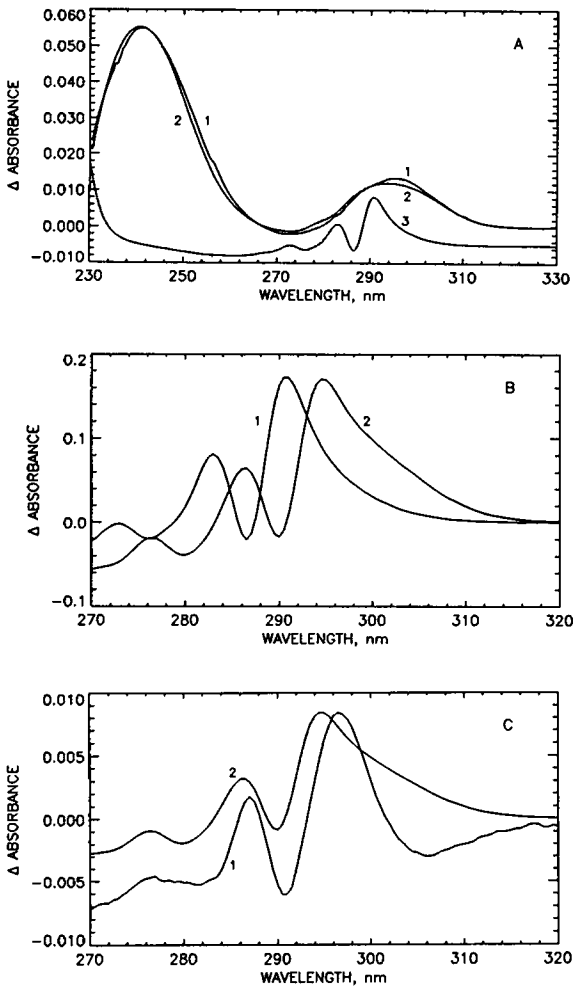

FIGURE 10 (*A*) Difference absorption spectra corresponding to: 1, deprotonation of tyrosine residue in water, pH 11 − 7; 2, purple membrane in 167 mM KCl for pH 10.7 − 9.9; 3, the shift of the absorption bands of a solution of tryptophan in water pH 11 − 7. (*B*) Comparison of the difference spectra caused by the red shift of the absorption spectrum of the tryptophan in water (1), and in dimethylsulfoxide (2). (*C*) 1, difference spectrum "1–2" where 1 and 2 are the spectra shown in Fig. 9 *B*. 2, First derivative of the absorption spectrum of tryptophan in dimethyl sulfoxide multiplied by $-\lambda^2$ (curve simulating the red shift of the absorption spectrum, the same as *curve 2* in Fig. 10 *B*).

al., 1982; Hess and Kushmitz, 1982; Sabés et al., 1984; Roepe et al., 1987).

The absorption spectrum of tryptophan is sensitive to the polarity of the environment. In nonpolar media the spectrum shifts to longer wavelengths (Herskovits and Sorensen, 1968; Donovan, 1969*a*). Fig. 10 *B* shows a simulation of the difference spectra produced by a red shift of the absorption spectrum of Trp in water (*curve 1*) and in a relatively nonpolar solvent dimethyl sulfoxide (*curve 2*). The shift[1] was simulated by taking the first derivative of the Trp spectrum multiplied by $-\lambda^2$: $dA/d\nu = -(dA/d\lambda)\lambda^2$. The tryptophan difference bands are shifted 4 nm to the red in dimethyl sulfoxide compared to their positions in water. This result is in agreement with other data, indicating that most of the tryptophan residues in bR are in a nonpolar environment (Ovchinnikov et al., 1979; Sherman, 1981; Permyakov and Shnyrov, 1983; Henderson et al., 1990).

The two near UV peaks in the pH-induced difference spectrum "pH 9.9 − 7.4" in light-adapted purple membrane (at 287.5 and 296.5 nm) are close to the peaks at 286 and 295 nm of the model spectrum obtained as a red shift of the spectrum of tryptophan in dimethyl sulfoxide (Fig. 10 *B*). This suggests that the increase in the pH from 7.4 to 9.9 causes a shift of the absorption band of tryptophan residues, localized in a nonpolar environment, to longer wavelengths. It is interesting that similar absorption changes accompany the bR to K conversion at low temperature (Efremov et al., 1978; Balashov and Litvin, 1985; Rothschild et al., 1986). They are apparently caused by the ≈ 1 nm shift to the red of the absorption spectrum of a tryptophan residue due to the charge perturbation in the primary light reaction (Balashov et al., 1988). Bands in the light-induced FTIR difference spectra assigned to the perturbation of tryptophan residue(s) were affected by Trp86 → Phe substitution, indicating that probably Trp86 is most sensitive to the change in the electrostatic environment near the chromophore (Rothschild et al., 1989*a*).Two more Trp residues, Trp182 and Trp189 (in the retinal binding pocket, Rothschild et al., 1989*b*), may also be sensitive to the perturbation of the chromophore environment.

Whereas the peaks at 287 and 296 nm can be explained as most likely due to a red shift of the Trp absorption spectrum, the band at 238 nm cannot be explained in a similar manner because perturbation of tryptophan is not accompanied by any significant increase of absorbance ~240 nm (Donovan, 1969*a*; Herskovitz and Sorensen, 1968; Fig. 10 *A*, *curve 3*). Complete denaturation of proteins can cause a large increase in

[1]The shift of the spectrum to longer wavelengths by $\Delta\nu$ can be expressed as $A(\nu + \Delta\nu)$. The difference spectrum would be $\Delta A = A(\nu + \Delta\nu) - A(\nu)$. When $\Delta\nu$ is small it is equal to $dA/d\nu \cdot \Delta\nu$. Because our spectra were measured as a function of wavelength the last expression can be rewritten: $dA/d\lambda \cdot d\lambda/d\nu \cdot \Delta\nu$; $\lambda = \text{constant}/\nu$, $d\lambda/d\nu = \text{constant}/\nu^2 = -\text{constant} \cdot \lambda^2$. Finally the difference spectrum of a red shift would be: $-\text{constant}\ dA/d\lambda \cdot \lambda^2$. In a narrow range of wavelengths, the contribution from λ^2 can be neglected, and the first derivative of the spectrum taken with a minus is a good approximation to the difference spectrum produced by the red shift of an absorption band.

absorbance at 230 nm (Mora and Elödi, 1968; Donovan 1969*b*), but the changes reported here are at longer wavelengths and are reversible (<pH 11.5 the absorbance changes are reversed if the sample pH is lowered to ~7, except that there is a slight increase in scattering; >pH 11.5 a major part of the absorbance change can be reversed upon neutralization if the sample is not left at high pH for a long time; however, sample scattering increases significantly). The most straightforward interpretation of the absorption band at 238 nm is that it is caused by the deprotonation of a tyrosine residue. The shift of the UV absorption band of this tyrosinate from its usual position at 240.5–241 nm (in alkaline water solution) to 238 nm may be because the phenolic group of this tyrosine residue is located in a very polar environment. It is known that the absorption band of tyrosine shifts to longer wavelengths in a nonpolar environment and to shorter wavelengths in polar environment (Herskovits and Sorensen, 1968; Donovan, 1969*a*). Thus, we suggest that the difference spectrum 1 in Fig. 9 *B* is due to the deprotonation of a tyrosine residue in a very polar environment, probably Tyr 185 (Ovchinnikov et al., 1986; Rothschild et al., 1989*b*) or Tyr 57, and a small red shift of the absorption spectrum of a tryptophan residue(s) in a nonpolar environment (Trp 86, Trp182, and Trp189), apparently in response to the tyrosine ionization. Fig. 10 *C* shows the result of subtraction of tyrosinate minus tyrosine difference spectrum from spectrum 1 of Fig. 9 *B*. The spectrum obtained (Fig. 10 *C*, *curve 1*) is close to the spectrum of the red shift of the absorption spectrum of tryptophan in dimethyl sulfoxide (Fig. 10 *C*, *curve 2*), which is in agreement with the above interpretation.

(*b*) The absorption changes produced by the increase of pH from pH 9.9 to 10.7 have two peaks, 241.6 and 296 nm and a small shoulder at 288 nm (Fig. 9 *B*, *curve 2*). These changes are almost identical with the difference spectrum of tyrosine deprotonation in solution (compare Fig. 10 *A*, *curve 1* vs. *curve 2*), which indicates that deprotonation of a second tyrosine residue occurs in the purple membrane between pH 9.9 and 10.7 (in 167 mM KCl). The amplitude of the absorption changes at 241 nm corresponds to the deprotonation of 0.84 Tyr/bR. An estimation from the amplitude of the absorption changes at 296 nm shows that ~1.0 tyrosine residue deprotonates between pH 9.9 and 10.7 (see Table 1). The deprotonation of this tyrosine residue does not affect the visible spectrum of bR or that of any tryptophan residues which suggests that it is not near the retinal binding pocket. The most likely candidates are the residues located on the outer surface of the membrane, Tyr64, Tyr79, Tyr131, and Tyr133.

(*c*) An increase in pH >11 causes a further increase in the amplitude of the 240 and 300 nm bands. The amplitude of the 240 nm band at pH 11.7 indicates that a third tyrosine residue deprotonates at this pH. The absorption changes accompanying the deprotonation of the third Tyr (Fig. 9 *B*, *curve 3*) residue are somewhat different from those of the two previous tyrosines. In the difference spectrum between light-adapted purple membrane at pH 11.8 and 11.3 both of the main maxima are shifted to longer wavelengths, to 246.5 and 301.7 nm, which indicates that the deprotonation of a tyrosine residue located in a nonpolar environment occurs. The shoulder at 290.6 nm indicates the perturbation of tryptophan residues, apparently caused by a transition into a more polar environment.

Thus, the pH induced absorption changes in light-adapted purple membrane in 167 mM KCl indicate that three tyrosine residues deprotonate with different pK's in the pH range 9–11.75 (in 167 mM KCl). Deprotonation of one tyrosine residue with pK 9 correlates with the absorption changes at 630 nm, indicating that the transition into the alkaline purple form of bR, bR_a, is caused by the deprotonation of one tyrosine residue. Deprotonation of this tyrosine also causes a red shift of the absorption band of one or more tryptophan residues. The pK of the two other tyrosine residues are ~10.3 and 11.3 in 167 mM KCl.

Photoproducts P500 and P380

The pH-induced absorption changes described above were obtained in purple membrane suspensions light-adapted at neutral pH and then adjusted to a high pH in the dark. When the purple membrane was light-adapted a second time at high pH the formation of a species absorbing at 380 nm in addition to the bR_a and P480 nm is observed. Fig. 11 *A* shows absorption spectra of a suspension of light-adapted purple membrane (in 0.2 M KCl) measured in the pH range from pH 7 to 11.4 at 20°C. The difference spectrum "pH 9.6 minus pH 7" (Fig. 11 *B*) has a positive band at 630 nm, a negative band at 568 nm, a wide positive band with a maximum around 380 nm, and sharp peaks at 299 nm (main) and 288 nm. With further increases in pH the band with a maximum at 380 nm increases in amplitude, whereas the 630 nm band becomes less prominent (Fig. 11 *C*). At pH >12 a positive band with its maximum at 460–470 nm appears which is mainly due to P480.

The reactions which resulted in the formation of a photoproduct absorbing at 380 nm were revealed by using long wavelength illumination and low temperatures for better separation and stabilization of the photoproducts. Fig. 12 *A*, curve 1 shows the absorption spectrum of a water glycerol (1:2) suspension of purple membrane, (pH 9.9, 0.4 M KCl) which was light-adapted at 20°C and then cooled to 10°C to slow down the

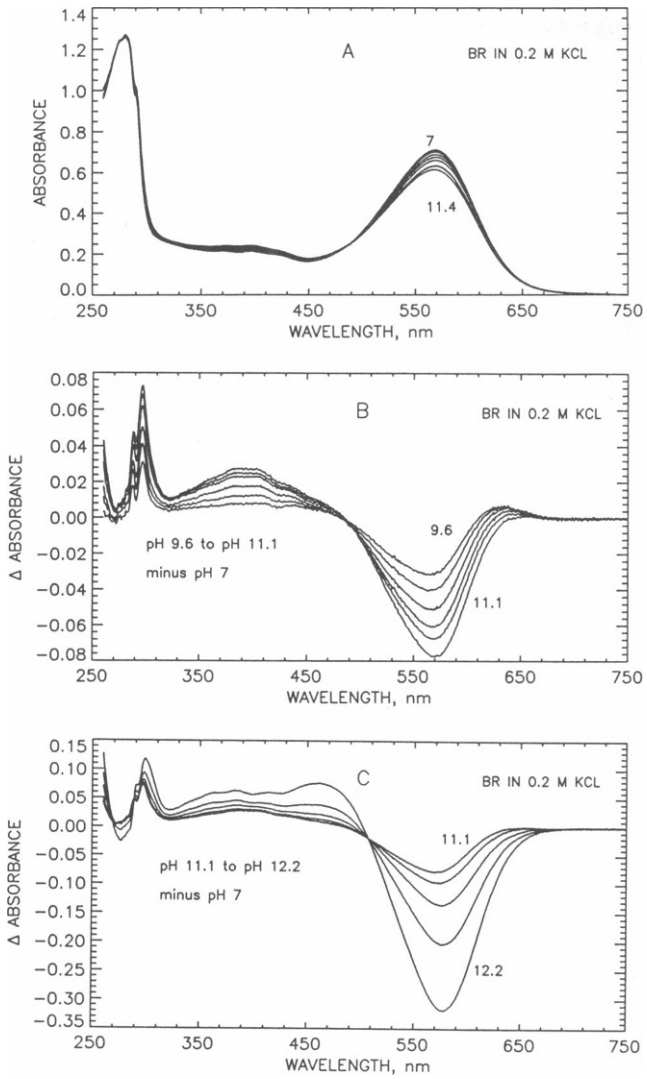

FIGURE 11 Absorption changes produced by increasing the pH of a suspension of light-adapted purple membrane. (*A*) Absorption spectra at pH 7, 8.4, 9.2, 9.7, 10.3, 11.4. (*B*) Difference spectra pH_i − pH 7; pH_i 9.6, 9.7, 9.9, 10.3, 10.8, 11, 11.1. (*C*) Difference spectra pH_i − pH 7; pH_i 11.1, 11.4, 11.8, 12, 12.2. Purple membranes were light-adapted with orange light (> 520 nm) after each pH adjustment.

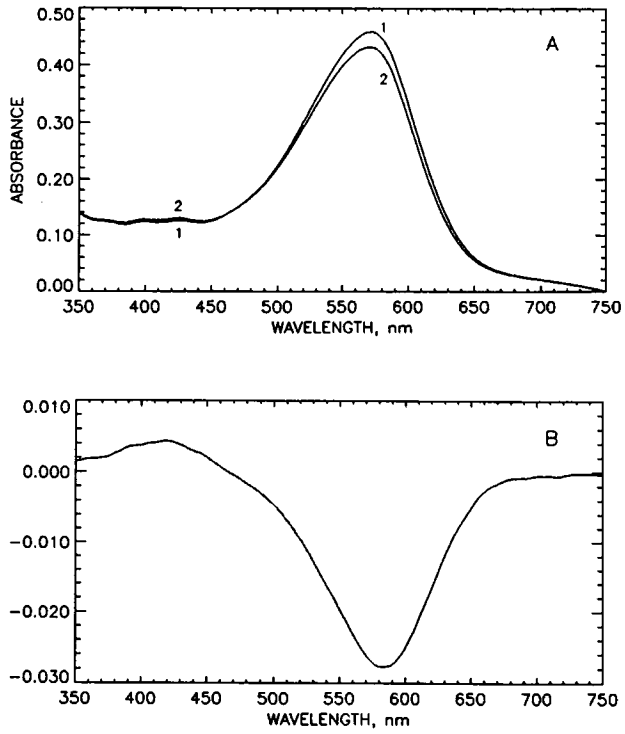

FIGURE 12 (*A*) 1, Suspension of purple membrane (pH 9.9, 0.4 M KCl, light-adapted at 20°C and cooled to 10°C); 2, after illumination at 620 nm. (*B*) Difference spectrum 2 − 1.

processes of dark adaptation. Illumination of this sample at 620 nm causes a decrease in the absorbance of the main band and an increase in absorbance at 450 nm (Fig. 12 *A*, *curve 2*). The difference absorption spectrum (2-1; Fig. 12 *B*) shows that the photoproduct has a smaller extinction and absorbs at shorter wavelengths. Illumination at 620 nm did not cause the formation of the 380 nm absorbing species which indicates that it is not formed directly from bR.

Further cooling of the sample to −60°C and illumination at 580 nm produces absorbance changes peculiar to the transformation of *trans*-bR into the M intermediate (Fig. 13, *curve 2*). Subsequent illumination of this sample at shorter wavelengths, 500 nm, produces additional absorbance changes which are characterized by a negative band at 500 nm and a positive band at 382 nm (Fig. 13, *curve 2*). Illumination at 370 nm reverses these changes. Warming the sample up to room temperature results in the disappearance of the 382 and 500 nm photoproducts.

These observations indicate that upon illumination at high pH part of the bR (apparently the alkaline form of bR, bR_a) is transformed into a long-lived photoproduct with its absorption maximum at ~500 nm (P500). This photoproduct in turn is photoactive and under illumination is converted into a state with an absorption maximum at ~380 nm (P380). When light adaptation is produced by white or yellow light (> 500 nm) P500 does not accumulate in the sample because it is converted into P380 (see the difference spectra shown in Fig. 11 *B*). Illumination at 380 nm converts P380 back into P500. At temperatures > 0°C, P380 converts in the dark to bR (perhaps due to a decrease in pH upon increase in temperature, see Methods). The pathways of interconversions of bR_a, P500, and P380 are shown in the scheme in Fig. 13 *B*. This scheme is in agreement with earlier

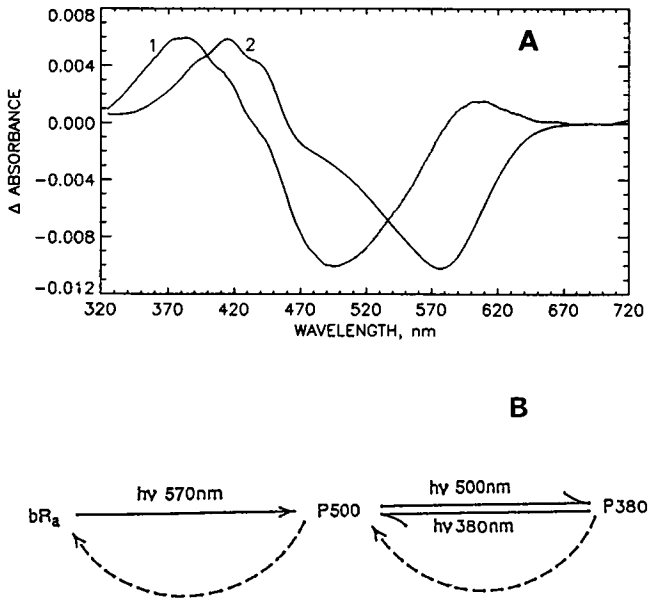

FIGURE 13 (*A*) Difference absorption spectra accompanying photoconversion of bR into M (1) and P500 into P380 (2). These difference spectra were produced by cooling sample 2 in Fig. 12 to −60°C and illuminating at: 1, 580 nm; 2, subsequent illumination at 500 nm; (*B*) scheme of light reactions yielding intermediates P500 and P380 at high pH.

observations of Shkrob and Rodionov (1978) and Druckman et al. (1982) who mentioned that illumination of bR at high pH facilitates the formation of a species absorbing at 370–380 nm. Also a recent study of Kaulen et al. (1990) describes a long-lived spectral relaxation of purple membrane in proteoliposomes at high pH induced by a 532 nm laser flash which was characterized by a positive band at 390 nm and a negative band at 460 nm. Illumination of P500 produces an M-like species (P380) which is different from M412. The place of P500 in the scheme of the reactions of the alkaline bR is unknown. It can be an intermediate of the photocycle or a product of a side reaction. It is interesting to note that the photoreversible reactions of P500 ⇔ P380 resemble the reactions of visual rhodopsin and its Meta-II intermediate.

DISCUSSION

We have found that at high pH the absorption spectrum of a light-adapted suspension of the purple membrane shifts ≈ 1.5 nm to the longer wavelengths. The pK of the red shift is salt dependent; the intrinsic pK being ~8.3. This finding has several aspects.

Acid-base equilibrium and two species of all-*trans*-bR at high pH

The coincidence of the pK of the red shift with (*a*) the pK of the increase in the amplitude of fast M formation, and (*b*) the pK of tyrosine deprotonation provide strong evidence that the deprotonation of a tyrosine residue with an intrinsic pK 8.3 (8.5 at high salt concentration and ~10.9 in water) is the cause of the red shift of the absorption band and the increase in the amplitude of fast M formation. Thus in the pH range from 8 to 10 (in high salt) and 9–11 (in low salt suspensions) bacteriorhodopsin in light-adapted purple membrane exists in two forms, "neutral" bR, and "alkaline" bR$_a$ (see Table 1). The two bR's are in a salt- and pH-dependent equilibrium: bR ⇔ bR$_a$. Flash excitation of these pigments results in photocycles which differ in the rate of deprotonation of the Schiff base during the L to M transition.

Our finding that three distinct tyrosines deprotonate with pK's in 167 mM KCl of ~9.0, 10.3, and 11.3 is in apparent disagreement with the recent studies of Herzfeld et al. (1990) and Ames et al. (1990). These authors using NMR and resonance Raman spectroscopy find no tyrosines deprotonating in the dark in bR in 100 mM borate or carbonate buffers (Herzfeld et al., 1990) or 200 mM NaCl (Ames et al., 1990) at pH's below 11.5 or 12. We have no explanation for this discrepancy.

Problem of single or parallel photocycles

In recent papers on the kinetics of the bR photocycle (Ames and Mathies, 1990; Varo and Lanyi, 1990), schemes containing parallel photocycles are discussed as less suitable alternatives to schemes of only one cycle with back reactions at certain points which enable a better fit of the multicomponent formation and decay of M (Parodi et al., 1984). In these schemes the rate constants of the L to M transition and the back reaction are pH dependent in the alkaline pH range. The nature of this pH dependence remains unclear in the framework of these schemes. The description of the kinetics of bR photocycle at high pH in terms of parallel photocycles does not contradict the idea of back reactions as an essential feature of many of the dark reactions of the photocycle (Parodi et al., 1984; Chernavskii et al., 1989; Otto et al., 1989; Ames and Mathies, 1990; Varo and Lanyi, 1990) but instead supplements it. It accounts for the existence of an alkaline form of bR, and the importance of acid-base transitions of certain amino acids in the functioning of bacteriorhodopsin. It also accounts for the existence of two major kinetic components (slow, 84 μs, and fast, 6 μs + 0.4 μs) in the L to M

transition at high pH whose rate constants do not depend on pH, but whose amplitudes do (Hanamoto et al., 1984; Liu, 1990).

Tyrosine participation in the photoreactions of bR

The finding of the alkaline form of bR, bR_a, and its coupling to tyrosine deprotonation and fast M formation gives support to the idea of two different bR species at high pH suggested in the papers of Hanamoto et al. (1984), Corcoran et al. (1986), Dancshazy et al. (1988), Diller and Stockburger, (1988), Govindjee et al. (1989), and Bitting et al. (1990).

There are at least three hypotheses that can be invoked to connect our data with the possible involvement of tyrosines in the photocycle. The first one is that the tyrosine residue does not deprotonate during the photocycle, but deprotonation of a tyrosine residue at high pH in the dark results in changes of protein-chromophore electrostatic interactions, which in turn cause an increase in the amplitude of the fast component of M formation, chromophore, and tryptophan absorption shift. In this minimum scheme the tyrosine residue does not play any specific role but causes an "electrostatic perturbation" of the kinetics of the cycle at high pH.

A second hypothesis is that the deprotonation of tyrosine at high pH changes not only the kinetics of L to M transition but also the pathway of the proton. Evidence for this is the decrease in the photoelectric current associated with the proton release (B2) at high pH (Liu, 1990).

The third possibility is that deprotonation of the tyrosine occurs not only at high pH in the dark but also during the photocycle at neutral pH. In particular our data are in agreement with the model of Kalisky et al. (1981), Hanamoto et al. (1984), Ovchinnikov et al. (1986), and Roepe et al. (1987), which propose that the protonation state of a tyrosine residue controls the rate of Schiff base deprotonation during M formation. Indeed, Hanamoto et al. (1984) have shown that the light induced change at 297 nm has a pK of ~9.6 in 100 mM NaCl and 8.6 in 4 M NaCl (Dupuis and El-Sayed, 1985), almost the same as the pK of the pH induced changes we report here. As noted above, these light induced changes in the UV are probably due to the deprotonation of a tyrosine and the subsequent electrostatic pertubation of tryptophans. However, the exact shape of the pH induced and light induced changes in the UV are somewhat different which may reflect contributions from the chromophore, electrostatic pertubations due to deprotonation of the Schiff base and protonation of the acceptor group (Asp85, see Butt et al., 1989) or other changes in the protein. Nevertheless it seems possible or even likely that the tyrosine deprotonated as a consequence of light absorption is the same rather unusual, low pK, tyrosine which we have suggested is deprotonated in the dark.

Two specific models can be suggested incorporating light induced tyrosine deprotonation during the formation of M. The first is based on the proposal of Kalisky et al. (1981) and Rosenbach et al. (1982) that the deprotonation of a tyrosine is a rate limiting step in the L to M transition. The following scheme can be suggested. The state of a tyrosine residue controls the deprotonation of the Schiff base. The transfer of a proton from the Schiff base to Asp85 becomes possible only if the tyrosine has first been deprotonated. The 85 μs component of the photocurrent signal at neutral pH (B2 component) (Liu, 1990) may then correspond both to the deprotonation of tyrosine and the transfer of a proton to Asp85. At high pH the tyrosine residue is already deprotonated and the fast 0.4 and 6 μs components of B2 signal correspond to deprotonation of the Schiff base and protonation of Asp85 only. The decrease in the area of the B2 photocurrent signal (which is proportional to the number of molecules undergoing the transition and the distance of the charge displacement) at high pH would indicate a change in the pathway of the proton. Hanamoto et al. (1984) have explicitly rejected such schemes because they find that the rate of the light induced change at 297 nm, presumably reflecting the tyrosine ionization, is about the same or somewhat slower than the normal (slow) rate of M formation. However, the complex nature of the change at 297 nm has not yet been fully clarified.

A second model incorporating light induced tyrosine deprotonation in the photocycle would place the tyrosine later in the proton release path. In this model the proton would normally first go to the primary acceptor upon deprotonation of the Schiff base, Asp85, in a time of the order of 85 μs at room temperature, neutral pH (Siebert et al., 1982; Butt et al., 1989). The appearance of the proton on Asp85 would then cause the tyrosine to deprotonate, probably leading to the proton appearing in solution at about the same time that the Schiff base deprotonated, but while the Asp85 remained protonated (Siebert et al., 1982; Liu, 1990). If this is the same tyrosine that is deprotonated at high pH in the dark, then the light induced path of the proton at high pH may well be different, the more rapid formation of M being due to the now deprotonated tyrosine affecting the rate at which the primary acceptor receives the proton. We also suggest that, because the tyrosine is already deprotonated, the protonation of the Asp85 acceptor cannot cause the tyrosine to expel its proton into solution. This

hypothesis would be consistent with the observation of Liu (1990) and also Li et al. (1984) that the quantum efficiency for proton expulsion for single turnover flashes drops off considerably at high pH. Thus, it probably is worthwhile to recheck the proton pumping data for tyrosine to phenylalanine mutants (Mogi et al., 1988) under single turnover conditions; interestingly Soppa et al. (1988) found that a tyrosine 57 to Asn mutant of bR had no proton pumping activity.

At present there is no way to unequivocally distinguish between the various hypotheses outlined above concerning the involvement of tyrosine deprotonation in the photocycle and proton pumping activity of purple membrane.

Two more tyrosine residues deprotonate upon increasing the pH from 10 to 11.8. At present it is not clear whether they affect the rate and efficiency of the photochemical reaction and proton transfer in bR. If they do, several alkaline forms of bR should be discriminated, bR'_a, bR''_a, bR'''_a, having respectively one, two, or three tyrosine residues in the deprotonated state.

On the nature of the chromophore and tryptophan absorption shift: electrostatic interactions in the purple membrane and charge separation upon excitation of bacteriorhodopsin

Upon raising the pH the chromophore absorption band of bR shifts as a whole without a significant change in its shape and half-width. There are two possible origins for this shift. One is that it is due to a pH induced conformational change. In most models of color regulation, the position of the absorption band in bacteriorhodopsin is mainly determined by the structure of a complex consisting of a protonated Schiff base connected through a weak hydrogen bond to the counter ion with other external charges modulating this interaction (Nakanishi et al., 1980; Spudich et al., 1986). Small changes in the distance between the Schiff base and the counter ion will greatly affect the absorption maximum of the pigment. Thus, the small shift of the absorption spectrum at alkaline pH may be explained by a minor conformational change of the protein. At present this hypothesis cannot be proven or rejected. A second possibility, which seems perhaps more likely and more interesting to discuss, is that the shift of the absorption spectrum is electrochromic in nature, being caused by the appearance of an electric charge in the protein near the chromophore. This negatively charged group (a deprotonated tyrosine residue) will produce an electrostatic field, which will cause a shift of the absorption spectrum due to the Stark (or electrochromic) effect. To the first approximation the direction and the magnitude of the shift depends on the strength of the field, **F**, the change in the electric dipole moment, $\Delta\mu$, and polarizability, $\Delta\alpha$:

$$h\Delta\nu = \mathbf{F}\Delta\mu, - 1/2\Delta\alpha\mathbf{F}^2,$$

where h is the Plank constant and $\Delta\nu$ is the shift of absorption band. According to the recent measurements of Birge and Zhang (1990), the change of the dipole moment of bacteriorhodopsin is 13.5 D, with the electron density shifting towards the Schiff base upon excitation (Birge, 1990). This dipole moment change will result in a blue shift if the charge is near the Schiff base, in no shift if it is near the center of the chromophore, and in a red shift if it is near the ionone ring. We assume that the negative charge is in the vicinity of the Schiff base to explain its effect on the deprotonation of the Schiff base. Thus there should be a blue shift of the absorption band due to the dipole moment change. The polarizability change, unknown for the bacteriorhodopsin chromophore, will always cause a red shift independent of the position of the charge. We propose that the red shift is most easily explained by a negative charge (tyrosinate) located near C12-C11, so that it is close to the Schiff base but does not give a large blue shift, and that the chromophore has a large polarizability change upon excitation of the order of 400 $Å_3$, leading to the net red shift.

If we assume that the absorbance changes at 615 nm are due to an electrochromic effect, then it is interesting that the maximum amplitude did not show a strong dependence on salt concentration. It was equal to 80, 76, 91, and 71 relative units in the presence of 0.0005, 0.02, 0.2 and 1.5 M KCl, respectively. This indicates that if the shift is produced directly by the field which appears upon the dissociation of a group, this group is inaccessible to cations. This suggests that the group is buried inside the protein, because otherwise the increased salt concentration would be expected to decrease the size of the electric field produced by the charged group and thus reduce the size of the effect.

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REFERENCES

Aldashev, A. A., and E. S. Efremov. 1983. State of the tyrosine and tryptophan residues in bacteriorhodopsin and bacterioopsin. *Bioorg. Khim.* 9:11–25.

Ames, J. B., and R. A. Mathies. 1990. The role of back-reactions and proton uptake during the N → O transition in bacteriorhodopsin's photocycle: a kinetic resonance Raman study. *Biochemistry.* 29:7181–7190.

Ames, J. B., S. R. Bolton, M. M. Netto, and R. A. Mathies. 1990. Ultraviolet resonance Raman spectroscopy of bacteriorhodopsin: evidence against tyrosinate in the photocycle. *J. Am. Chem. Soc.* 112:9007–9009.

Balashov, S. P., and F. F. Litvin. 1985. Photochemical conversions of bacteriorhodopsin. A. A. Krasnovsky, editor. Moscow State University Press. 123 pp.

Balashov, S. P., N. V. Karneeva, F. F. Litvin, and V. A. Sineshchekov. 1987. Primary photoreactions of *trans*- and 13-*cis*-bacteriorhodopsin. Retinal Proteins. Yu. A. Ovchinnikov, editor. VNU Science Press BV (VSP) Utrecht, Netherlands. 505–517.

Balashov, S. P., F. F. Litvin, and V. A. Sineshchekov. 1988. Photochemical processes of light energy transformation in bacteriorhodopsin. Physicochemical Biology Reviews. Vol. 8. V. P. Skulachev, editor. Harwood Academic Publishers GmbH, Chur, Switzerland. 1–61.

Becher, B., and J. Y. Cassim. 1975. Improved isolation procedures for the purple membrane of *Halobacterium halobium*. *Prep. Biochem.* 5:161–178.

Bitting, H. C., D.-J. Jang, and M. A. El-Sayed. 1990. Multiple cycles of bacteriorhodopsin at high pH. *Photochem. Photobiol.* 51:593–598.

Birge, R. R. 1990. Photophysics and molecular electronic applications of the rhodopsins. *Annu. Rev. Phys. Chem.* 41:683–733.

Birge, R. R., and C.-F. Zhang. 1990. Two-photon double resonance spectroscopy of bacteriorhodopsin. Assignment of the electronic and dipolar properties of the low-lying $^1A_g^{*-}$ and $^1B^{u*+}$-like π, π^* states. *J. Chem. Phys.* 92:7178–7195.

Bogomolni, R. A. 1980. Conversion of light energy into a proton electrochemical potential by bacteriorhodopsin. Bioelectrochemistry. Plenum Publishing Corp., New York. 83–95.

Bogomolni, R. A., R. Renthal, and J. K. Lanyi. 1978. Intramolecular proton transfer during the bacteriorhodopsin photochemical cycle. *Biophys. J.* 21:183a. (Abstr.)

Bücher, H., and H. Kuhn. 1970. Difference between ground and excited state dipole moments and polarizabilities as determined from electrochromism of Scheibe-dye-aggregates in monolayer assemblies. *Z. Naturforsch. Sect. B Chem. Sci.* 25b:1323–1327.

Butt, H. J., K. Fendler, E. Bamberg, J. Tittor, and D. Oesterhelt. 1989. Aspartic acids 96 and 85 play a central role in the function of bacteriorhodopsin as a proton pump. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1657–1663.

Chernavskii, D. V., I. V. Chizov, R. H. Lozier, T. M. Murina, A. M. Prokhorov, and B. V. Zubov. 1989. Kinetic model of bacteriorhodopsin photocycle: pathway from M state to bR. *Photochem. Photobiol.* 49:649–653.

Corcoran, T. C., P. Dupuis, and M. A. El-Sayed. 1986. The effect of ionic strength and pH on the protonated Schiff base and tyrosine deprotonation kinetics during the bacteriorhodopsin photocycle. *Photochem. Photobiol.* 43:655–660.

Czégé, J., A. Dér, L. Zimányi, and L. Keszthelyi. 1982. Restriction of motion of protein side chains during the photocycle of bacteriorhodopsin. *Proc. Nat. Acad. Sci. USA.* 79:7273–7277.

Dancshazy, Zs., R. Govindjee, and T. G. Ebrey. 1988. Independent photocycles of the spectrally district forms of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 85:6358–6361.

Diller, R., and M. Stockburger. 1988. Kinetic resonance Raman studies reveal different conformational states of bacteriorhodopsin. *Biochemistry.* 27:7641–7651.

Donovan, J. W. 1969*a*. Ultraviolet absorption. *In* Physical Principles and Techniques of Protein Chemistry. Part A. S. J. Leach, editor. Academic Press, New York. 101–169.

Donovan, J. W. 1969*b*. Changes in ultraviolet absorption produced by alteration of protein confirmation. *J. Biol. Chem.* 211:1961–1967.

Druckman, S., M. Ottolenghi, A. Pande, J. Pande, and R. H. Callender. 1982. Acid-base equilibrium of the Schiff Base in Bacteriorhodopsin. *Biochemistry.* 21:4953–4959.

Dupuis, P., and M. A. El-Sayed. 1985. Effect of salt on the tyrosine and protonated Schiff base deprotonation kinetics in bacteriorhodopsin. *Can. J. Chem.* 63:1699–1704.

Efremov, E. S., A. I. Sotnichenko, A. A. Aldashev, A. M. Surin, and A. I. Miroshnikov. 1978. Tyrosyl and tryptophyl residues in bacteriorhodopsin: spectral study and chemical modification. *Proc. FRG-USSR Symp. Chem. Pept. Proteins. Second. Munich.* 71–73.

Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh. 1966. Hydrogen ion buffers for biological research. *Biochemistry.* 5:467–477.

Govindjee, R., Zs. Dancshazy, and T. G. Ebrey. 1989. Difference spectra of late intermediates of the bacteriorhodopsin photocycle. *Proc. Int. Soc. Opt. Eng. Biomol. Spectrosc.* 1057:126–137.

Hanamoto, J. H., P. Dupuis, and M. A. El-Sayed. 1984. On the protein (tyrosine)-chromophore (protonated Schiff base) coupling in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 81:7083–7088.

Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* 213:899–929.

Herskovits, T. T., and M. Sorensen. 1968. Studies of tyrosyl and tryptophyl residues in proteins. I. Solvent perturbation data of model compounds. *Biochemistry.* 7:2523–2532.

Herzfeld, J., S. K. DasGupta, M. R. Farrar, G. S. Harbison, A. E. McDermott, S. L. Pelletier, D. P. Raleigh, S. O. Smith, C. Winkel, J. Lugtenburg, and R. G. Griffin. 1990. Solid state ^{13}C NMR study of tyrosine protonation in dark-adapted bacteriorhodopsin. *Biochemistry.* 29:5567–5574.

Hess, B., and D. Kuschmitz. 1979. Kinetic interaction between aromatic residues and the retinal chromophore of bacteriorhodopsin during the photocycle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100: 334–340.

Holz, M., L. A. Drachev, T. Mogi, H. Otto, A. D. Kaulen, M. P. Heyn, V. P. Skulachev, and H. G. Khorana. 1989. Replacement of aspartic acid-96 by asparagine in bacteriorhodopsin slows both the decay of the M intermediate and the associated proton movement. *Proc. Natl. Acad. Sci. USA.* 86:2167–2171.

Honig, B., and T. G. Ebrey. 1974. The structure and spectra of the chromophore of the visual pigments. *Annu. Rev. Biophys. Bioeng.* 3:151–177.

Jonas, R., Y. Koutalos, and T. G. Ebrey. 1990. Purple membrane: surface charge density and multiple effects of pH and cations. *Photochem. Photobiol.* 52:1163–1177.

Kalisky O., M. Ottolenghi, B. Honig and R. Korenstein. 1981. Environmental effects and photoreaction of the M_{412} photoproduct of bacteriorhodopsin: implication for the mechanism of proton pumping. *Biochemistry.* 20:649–655.

Kaulen, A. D., L. A. Drachev, and V. V. Zorina. 1990. Proton transport and M-type intermediate formation by 13-*cis*-bacteriorhodopsin. *Biochim. Biophys. Acta.* 1018:103–113.

Koutalos, Y., T. G. Ebrey, H. R. Gilson, and B. Honig. 1990. Octopus photoreceptor membranes. Surface charge density and pK of the Schiff base of the pigments. *Biophys. J.* 58:491–501.

Li, Q.-Q., R. Govindjee, and T. G. Ebrey. 1984. A correlation between proton pumping and the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 81:7079–7082.

Litvin, F. F., S. P. Balashov, and V. A. Sineshchekov. 1975. Study of the primary photochemical conversions of bacteriorhodopsin in purple membranes and cells of *Halobacterium halobium* by the low temperature spectrophotometry. *Bioorg. Chem.* 1:1767–1777.

Liu, S. Y. 1990. Light-induced currents from oriented purple membrane: I. Correlation of the microsecond component (B2) with the L-M photocycle transition. *Biophys. J.* 57:943–950.

Maeda, A., M. Nakagawa, T. Ogura, and T. Kitagawa. 1988. Molecular Physiology of Retinal Proteins. T. Hara, editor. 85–90.

Maeda, A., T. Ogura and T. Kitagawa. 1986. Resonance Raman study on proton-dissociated state of bacteriorhodopsin: stabilization of L-like intermediate having the all-*trans* chromophore. *Biochemistry.* 25:2798–2803.

Mathies, R., and L. Stryer. 1976. Retinal has a highly dipolar vertically excited singlet state: implications for vision. *Proc. Natl. Acad. Sci. USA.* 73:2169–2173.

Mathies, R. A., C. H. Brito Cruz, W. T. Pollard, and C. V. Shank. 1988. Direct observation of the femtosecond excited-state *cis-trans* isomerization in bacteriorhodopsin. *Science.* (Wash. DC.) 240:777–779.

Maurel, P., G. H. B. Hoa, and P. Douzou. 1975. The pH dependence of the tryptic hydrolysis of benzoyl-L-arginine ethyl ester in cooled mixed solvents. *J. Biol. Chem.* 250:1376–1382.

Mogi, T., L. J. Stern, N. R. Hackett, and H. G. Khorana. 1987. Bacteriorhodopsin mutants containing single tyrosine to phenylalanine substitutions are all active in proton translocation. *Proc. Natl. Acad. Sci. USA.* 84:5595–5599.

Mora, S., and P. Elödi. 1968. Investigation of the near and far ultraviolet denaturation difference spectra of dehydrogenases. *Eur. J. Biochem.* 5:57–582.

Muccio, D. D., and J. Y. Cassim. 1979. Interpretations of the effects of pH on the spectra of purple membane. *J. Mol. Biol.* 135:595–609.

Nakanishi, K., V. Balogh-Nair, M. Arnaboldi, K. Tsujimoto, and B. Honig. 1980. An external point charge model for bacteriorhodopsin to account for its purple color. *J. Am. Chem. Soc.* 102:7945–7947.

Ort, D. R., and W. W. Parson. 1978. Flash-induced volume changes of bacteriorhodopsin-containing membrane fragments and their relationship to proton movements and absorbance transients. *J. Biol. Chem.* 253:6158–6164.

Otto, H., T. Marti, M. Holz, T. Mogi, M. Lindau, H. G. Khorana, and M. P. Heyn. 1989. Aspartic acid-96 is the internal proton donor in the reprotonation of the Schiff base of bacteriorhodopsin. *Biochemistry.* 86:9228–9232.

Ovchinnikov, Yu. A., A. M. Shkrob, and A. V. Rodionov. 1980. Spectral transition of bacteriorhodopsin as an indicator of its state in purple membranes. *Bioorgan. Chem.* 6:1483–1503.

Ovchinnikov, Yu. A., N. G. Abdulaev, A. V. Kiselev, I. R. Nabiev, H. V. Vasileva, and R. G. Efremov. 1986. Tyrosine residue catalyzes light-induced deprotonation of retinal aldimine in bacteriorhodopsin. *Biol. Membr.* 3:325–338.

Ovchinnikov, Yu. A., N. G. Abdulaev, M. Y. Feigina, A. V. Kiselev, and N. A. Lobanov. 1979. The structural basis of the functioning of bacteriorhodopsin: an overview. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:219–224.

Parodi, L. A., R. H. Lozier, S. M. Bhattacharjee, and J. F. Nagle. 1984. Testing kinetic models for the bacteriorhodopsin photocycle—II. Inclusion of an O to M backreaction. *Photochem. Photobiol.* 40:501–512.

Permyakov, E. A., and V. L. Shnyrov. 1983. A spectrofluorometric study of the environment of tryptophans in bacteriorhodopsin. *Biophys. Chem.* 18:145–152.

Roepe, P., P. L. Ahl, S. K. Das Gupta, J. Herzfeld, and K. J. Rothschild. 1987. Tyrosine and carboxyl protonation changes in the bacteriorhodopsin photocycle. 1. M_{412} and L_{550} intermediates. *J. Biochem.* 26:6696–6707.

Rosenbach, V., R. Goldberg, C. Gilon, and M. Ottolenghi. 1982. On the role of tyrosine in the photocycle of bacteriorhodopsin. *Photochem. Photobiol.* 36:197–201.

Rothschild, K. J., P. Roepe, P. L. Ahl, T. N. Earnest, R. A. Bogomolni, S. K. Das Gupta, C. M. Mulliken and J. Herzfeld. 1986. Evidence for a tyrosine protonation change during the primary phototransition of bacteriorhodopsin at low temperature. *Proc. Natl. Acad. Sci. USA.* 83:347–351.

Rothschild K. J., D. Gray, T. Mogi, T. Marti, M. S. Braiman, L. J. Stern, and H. G. Khorana. 1989*a*. Vibrational spectroscopy of bacteriorhodopsin mutants: chromophore isomerization perturbs tryptophan-86. *Biochemistry.* 28:7052–7059.

Rothschild, K. J., M. S. Braiman, T. Mogi, L. J. Stern, and H. G. Khorana. 1989*b*. Conserved amino acids in helix F of bacteriorhodopsin form part of retinal binding pocket. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 250:448–452.

Sabés, M., M. Duñach, J. Mañosa, A. Morros, and E. Padrós. 1984. Spectroscopic studies on the tryptophan residues of bacteriorhodopsin during the photocycle. *Photochem. Photobiophys.* 8:97–101.

Scherrer, P., and W. Stoeckenius. 1984. Selective Nitration of tyrosines-26 and -64 in bacteriorhodopsin with tetranitromethane. *Biochemistry.* 23:6195–6202.

Sherman, W. V. 1981. The ultraviolent fluorescence of bacteriorhodopsin and the location of tryptophanyl residues. *Photochem. Photobiol.* 33:367–371.

Shkrob, A. M., and A. V. Rodionov. 1978. Alkaline denaturation of bacteriorhodopsin in the purple membrane. *Bioorg. Chem.* 4:360–368.

Siebert, F., W. Mäntele, and W. Kreutz. 1982. Evidence for the protonation of two carboxylic groups during the photocycle of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Left.* 141:82–87.

Sineshchekov, V. A., S. P. Balashov, and F. F. Litvin. 1981. Fluorescence of photoactive bacteriorhodopsin. *Biophysics. (Engl. Transl. Biofiziko).* 26:986–995.

Soppa, J., J. Otomo, J. Straub, J. Tittor, S. Meessen, and D. Oesterhelt. 1989. Bacteriorhodopsin mutants of *Halobacterium* sp. GRB: II Characterization of mutants. *J. Biol. Chem.* 264:13049–13056.

Spudich, J. L., D. A. McCain, K. Nakanishi, M. Okabe, N. Shimizu, H. Rodman, B. Honig, and R. A. Bogomolni. 1986. Chromophore-protein interaction in bacteriorhodopsin. *Biophys. J.* 49:479–483.

Tokunaga, F., T. Iwasa, and T. Yoshizawa. 1976. Photochemical reaction of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 72:33–38.

Varo, G., and J. K. Lanyi. 1990. Pathways of the rise and decay of the M photointermediate(s) of bacteriorhodopsin. *Biochemistry.* 29:2241–2250.